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FINAL REPORT

Splenic Nerve Stimulation for Sepsis Model Development & Concept Evaluation

Study No.: 0001E0007

Study Director: Tina Billstrom

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Date

Physiological Research Laboratories
Division of Medtronic, Inc.
11520 Yellow Pine Street N.W.
Minneapolis, MN 55448

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APPENDICES

There are 26 pages to Appendix A.

I. INTRODUCTION

A. Purpose

On Redacted this protocol was initiated at Physiological Research Laboratories (located at 11520 Yellow Pine Str. NW, Minneapolis, MN 55448). This protocol was written to develop a sepsis model at PRL and to evaluate the ability of splenic nerve stimulation (SNS) to modulate a systemic immune response.

B. Study Design

In summary this study was divided into two phases.

Phase I: Three (3) animals were used to establish the porcine sepsis model. Each animal received LPS for two hours followed by electrical stimulation. Blood gases, body temp, arterial pressure, cardiac output, and blood draw were completed every ½ hour throughout the experiment.

Phase II: A total of thirteen (13) animals were used to investigate the effects of electrical stimulation on a sepsis model.

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II. STUDY OBJECTIVES

The study was designed to answer specific questions about the safety and efficacy of acute SNS to justify further investment in the project. The overall objectives of the study were to:

A. Evaluate the feasibility of splenic nerve electrical stimulation.
B. Evaluate any physiological changes resulting from the electrical stimulation.
C. Determine the effect of acute electrical stimulation on the LPS-induced inflammatory response, and blood chemistries.

III. TEST SYSTEMS

A. Species: Porcine

B. Number: 16

C. Weight Range: 40-65 kg

D. Sex: Male and Female

E. Age: Adult

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IV. RESULTS & CONCLUSIONS

All study objectives were met. There have been no significant deviations from the protocol that affected the study objectives.

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Elaboration of the objectives, including specific data and results are presented in the detailed Sponsor Final Report in Appendix A.

V. LIST OF PERSONNEL

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VI. STANDARDS

A. Facility

The Physiological Research Laboratories (PRL) of Medtronic, Inc. comply with the Animal Welfare Act of 1966 (P.L. 89-544), and all amendments. The Physiological Research Laboratories has been registered with the United States Department of Agriculture, Animal and Plant Health Services (Registration No. 41-51) since April 5, 1973. The Physiological Research Laboratories has maintained accreditation with the American Association for Accreditation of Laboratory Animal Care (AAALAC) since December 4, 1973.

B. Animal Care

Animals were selected following a prescribed quarantine. A staff veterinarian gave a complete physical exam to the animals within 72 hours of receipt.

Animals were cared for according to the Physiological Research Laboratories Standard Operating Procedures and The Guide for Care and Use of Laboratory Animals.

The animals were housed at PRL in cages that meet the weight-space specifications recommended in The Guide for the Care and Use of Laboratory Animals.

APPENDIX A:

Sponsor Final Report

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Medtronic®

Final Report

for

Splenic Nerve Stimulation for Sepsis Model
Development & Concept Evaluation
(Short Title: Splenic Nerve)

Study No.: 0001E0007
Study Director: Tina Billstrom
Study Sponsor: Lisa Shafer

Physiological Research Laboratories
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EXECUTIVE SUMMARY

Introduction:

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The research initiative is to determine the safety and therapeutic efficacy of acute electrical stimulation of the splenic nerve to treat sepsis. The purpose of the study is to explore the ability of electrical simulation of the splenic nerve to modulate the immune response elicited by a systemic bacterial infection. Redacted if electrical stimulation of the splenic nerve is capable of modulating a systemic immune response and reducing sepsis-induced mortality, there are several chronic applications where splenic nerve stimulation may also be beneficial. In conditions such as rheumatoid arthritis and other chronic inflammatory diseases, inflammatory processes may be modulated via stimulation of the splenic nerve. Redacted Redacted Instead of stimulating the vagus nerve to control the parasympathetic outflow stimulating the splenic nerve to control the sympathetic outflow may offer a more safe and efficacious approach to modulate an acute or chronic inflammatory condition.

The purpose of this acute study was to evaluate the ability of splenic nerve stimulation to modulate a systemic immune response.

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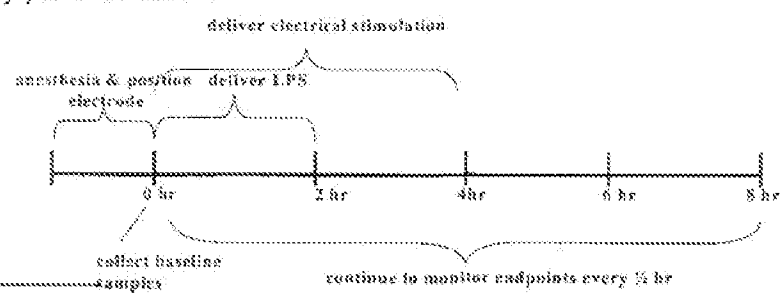
Objectives:

In addition to the preliminary goal of establishing a large animal sepsis model, the objectives of this study were:

A. Evaluate the feasibility of splenic nerve electrical stimulation.
B. Evaluate any physiological changes resulting from the electrical stimulation.
C. Determine the effect of acute electrical stimulation on the LPS-induced inflammatory response, and blood chemistries.

Study Design:

The procedure depicted below, with some variation, was conducted on seventeen pigs[1] in this non-GLP study. Sixteen of the procedures were performed under study protocol 0001E007 and one procedure was performed under study protocol S1604.



[1] Sixteen of the procedures were performed under study protocol 0001E007 and one procedure was performed under study protocol S1064. For the purpose of cohesive reporting, results from seventeen procedures are considered in this report.

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Animals were under anesthesia for the duration of the procedure. Sepsis was induced by continuous i.v. infusion of *Escherichia coli (E. coli)* lipopolysaccharide (LPS). Each animal had a sternotomy/laparatomy to implant a cuff electrode around a nerve as close to the splenic hilus as possible.
Blood gases, arterial blood, white blood cell count, blood pressure, cardiac output, and rectal temperatures were collected at baseline and at 30 minute intervals for the duration of the study. Cytokine analysis was performed on arterial blood samples. Animals were euthanized at the end of their procedure and taken to necropsy for evaluation.

Results:

- The pig was chosen as the appropriate animal model and surgical feasibility was established.
    - A dose of 5ug/kg/hour administered for two hours induces a sub-lethal sepsis response. This response was marked by a drop in blood pressure, decreased white blood cell count, a rise in blood lactate levels, a rise in pro-inflammatory cytokines and other measures.

- Electrical stimulation of the splenic nerve does not adversely affect cardiac physiology in this porcine sepsis model.
    - Blood pressure, blood gas values, oxygen status electrolyte values and metabolite values were not altered by electrical stimulation applied to the splenic nerve alone.

- Acute electrical stimulation of the splenic nerve prohibited the LPS induced increase in pro-inflammatory cytokines and the LPS induced decrease in WBC count (n=2) suggesting efficacy.

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Conclusions:
All study objectives were met. This study resulted in the establishment of a porcine sepsis model at Medtronic PRL. Changes in the white blood cell count and pro-inflammatory cytokine levels are consistent with rodent sepsis models. This large animal sepsis model allows for future investigation of therapies targeting inflammatory conditions and for pre-clinical evaluation of standard medical devices in such conditions.

Electrical stimulation of the splenic nerve is surgically feasible as it does not require a significantly invasive electrode placement in the pig. Furthermore, electrical stimulation of the splenic nerve does not appear to have adverse effects on tissue histology, blood chemistry and cardiac physiology.

Redacted **preliminary observations suggest that electrical stimulation may safely and effectively inhibit the inflammatory response.** Redacted

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KEY PERSONNEL

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I. BACKGROUND

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II. STUDY OBJECTIVE(S)

These studies were designed to answer questions concerning the safety and efficacy of acute splenic nerve stimulation to justify further investment in the project. The overall objectives of the study were to:

A. Evaluate the feasibility of splenic nerve electrical stimulation.
B. Evaluate any physiological changes resulting from the electrical stimulation.
C. Determine the effect of acute electrical stimulation on the LPS induced inflammatory response, and blood chemistries.

The goal of Phase I was to establish a large animal sepsis model to be used in the Phase II. The Phase I study had the following objectives:

- Identify a large mammal and establish an effective dose of LPS to generate a sub-lethal sepsis model.
- Evaluate the surgical feasibility of splenic nerve stimulation in the animal model.
- Evaluate any physiological/pathological changes resulting from the LPS or electrical stimulation (ES).

The goal of Phase II was to conduct a preliminary assessment of efficacy. Phase II had the following objectives:

- Determine the effect of acute SNS on the sepsis-induced inflammatory response compared to non-stimulated sepsis control animals.

III. STUDY DESIGN

This study was divided into two phases.
Phase I: Three (3) animals were used to establish the porcine sepsis model. Each animal received 5-50ug/kg/hr LPS for two hours. All three received some level of electrical stimulation within 10-100 Hz and all endpoints were monitored as described below.

Phase II: A total of fourteen (14) animals, were used to investigate the effects of electrical stimulation.
Table 1 describes the specific experimental effect applied.

IV. METHODS

This study used the porcine model of bacterial sepsis. Figure 1 demonstrates the basic sequence of events for each procedure. Specific methods are described in Appendix A. Briefly, animals were put under anesthesia. Following the induction of anesthesia, a left side incision to access the spleen was created. A cuff electrode (Fig. 2) was placed near the splenic nerve hilus (Fig. 3) for later use and baseline measurements were taken. Sepsis was induced by continuous i.v. infusion of Escherichia coli (E. coli) lipopolysaccharide in saline solution 5-50ug/kg/hr for two hours via a 12 cc leur-lock syringe and an infusion pump with catheter vis-à-vis the femoral vein. Animals then underwent acute, transient electrical stimulation to the splenic nerve or a sham operation. Pulses of electrical stimulation were applied to the splenic nerve for four to six hours. Electrical stimulation of the splenic nerve was achieved by a Medtronic test stimulator Model 3625. The cuff electrode was

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attached to the stimulator via alligator clip attaching the connector pin. Approximately 450 usec wide pulses and burst frequencies of about 10 Hz were used at 10 Volts (for specific stimulation parameter see Table 1).

The following data points were measured every half hour throughout the experiment.

1. Febrile response (Body temperatures via rectal thermometers)
2. Blood gases
3. Systemic arterial pressure
4. Cardiac Output/Pulmonary arterial pressure
5. White blood cell (WBC) count
6. Arterial blood draws for later determinations of concentrations of:
   a. TNFα (R&D Systems)*
   b. IL-6 (R&D Systems)*
   c. IL-10 (BioSource)*
   d. IL-1β (R & D Systems)*

* Denotes ELISA kits (Enzyme Linked Imuno-absorbant Assay). The ELISAs were conducted at Medtronic's Materials and Bioscience Center. Also a differential WBC analysis was drawn before and after infusion and at the end of the entire procedure, prior to euthanasia.

Figure 1. Diagram of acute study procedure.

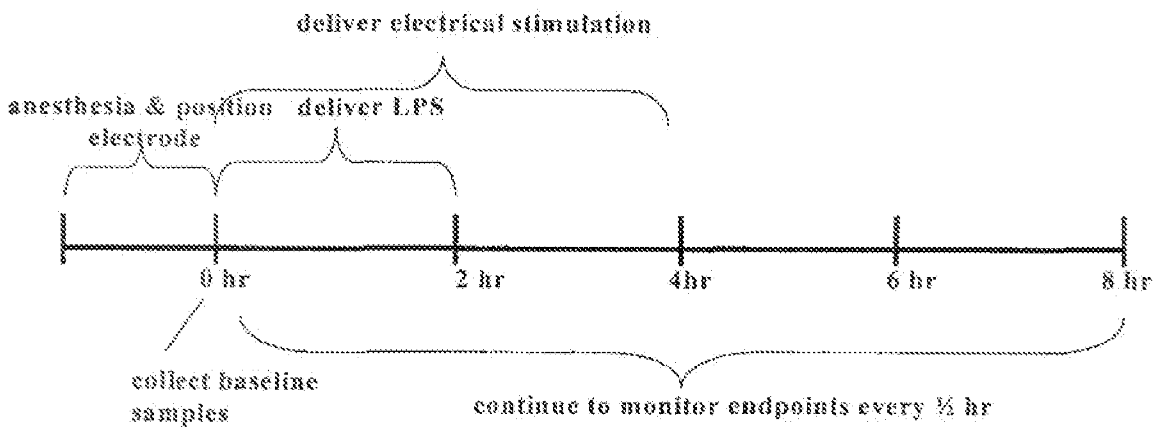
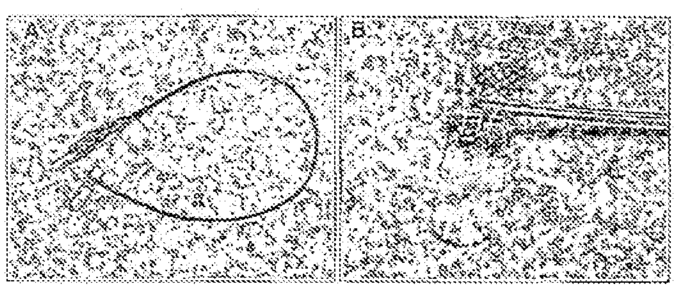


Figure 2. Cuff electrode used in study.



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V. RESULTS

Phase I: Establishing a porcine sepsis model

- *The pig was chosen as the appropriate animal model and surgical feasibility was established.*

Other large animal models were considered (canine, ovine) but the innervation of the porcine spleen, its size and it primary physiological function (non-contractile lymphoid organ) resembles that of a human's. Figure 3 demonstrates the relevant anatomy of the model. The target for stimulation is the nerves that reside in the splenic hilus. Since there are very few existing literature references pertaining to a porcine sepsis model, it was important to establish a model that met sub-lethal criteria.

Figure 3. Gross anatomy. A) Pig spleen in situ. B) Pig spleen, reflected. C) Splenic hilus.

A simple surgical approach was achieved by making a lateral flank incision. The head of the spleen was located and the nerve branches at the splenic hilus were bluntly dissected such that the mesh electrode could be passed underneath a nerve/connective tissue bundle. Often this bundle included vasculature in addition to nerve tissue. The spleen was then tucked back in situ and moistened gauze was placed over the incision for the duration of the procedure. Figure 4 demonstrates the surgical approach. Figure 4D demonstrates that after 6 hours of stimulation at various frequencies (1-100Hz) the artery and nerves remain intact.

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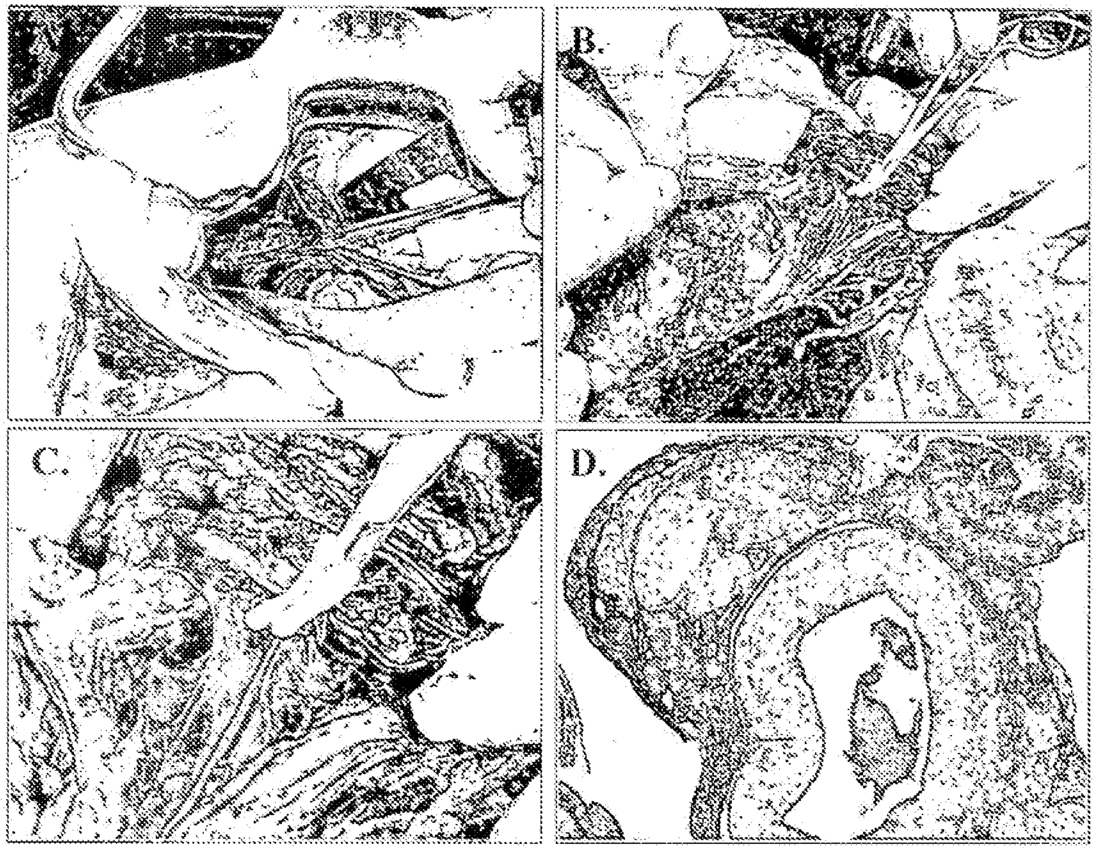
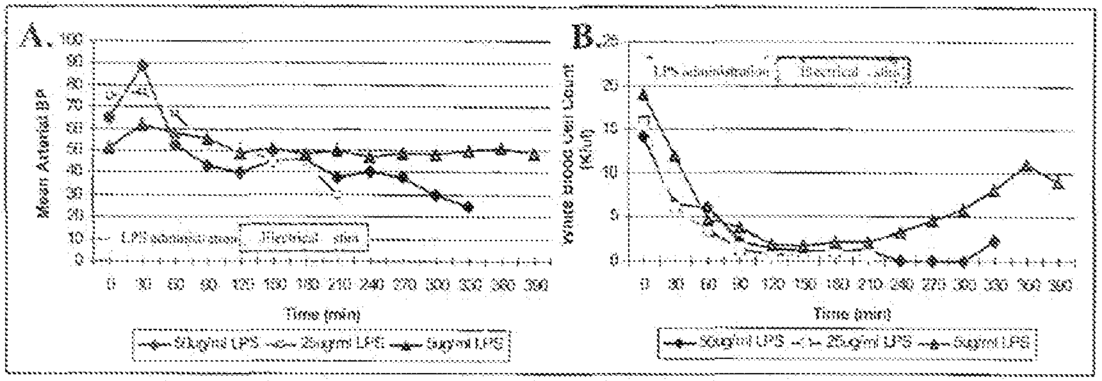
Figure 4. Surgical approach. A) Lateral flank incision. Cuff electrode was attached in the vicinity of scissor tips. B) Tissue reflected upon necropsy to demonstrate electrode placement. C) Higher magnification of B. Note branches of nerve and artery within cuff electrode. D) Histology of tissue under the cuff electrode.



- *5ug/kg/ml per hour induces a sub-lethal sepsis response.*

The administration of lipopolysaccharide is the most common sepsis model where the dose can be titrated per the desired level of severity. While mortality was not a practical endpoint for this study given ethical constraints, a sub-lethal dose (for an 8 hour procedure) was required. The defining features of a lethal sepsis model are a drop in mean arterial blood pressure (MABP), a sharp initial drop in white blood cell count (WBC), a rise in blood lactate levels, a rise in systemic Tumor Necrosis Factor (TNF) and other measures. At this dose, an animal that receives no sustaining measures (dobutamine, fibrillation, etc) would succumb 7-9 hours after the start of the procedure. Here, the animals that received a 50ug/ml/hr or a 25ug/ml/hr dose of LPS did not survive for the full 8-hour procedure. As shown in figure 5A, at a dose of 5ug/ml/hr for two hours the MABP can be stably maintained for the full 8 hours while still inducing a sub-lethal sepsis response as indicated by the drop in WBC count (Fig. 5B) and increase in lactate and TNF levels (data not shown).

Figure 5. LPS dose response. A) LPS effects on mean arterial blood pressure. B) LPS effects on white blood cell count.



- *Splenic nerve stimulation does not appear to adversely affect cardiac physiology.*

Given that the splenic nerve is a sympathetic nerve, it was important to demonstrate that electrical stimulation of the splenic nerve does not exert negative effects on cardiac physiology. Electrical stimulation was applied with varying frequencies from 1-100 hz (450usec pulse width, 10 mAmps) and no negative cardiac effects were observed. Blood gas values, oxygen status, electrolyte values and metabolite values were not altered by electrical stimulation (see archived blood chemistry printouts). Furthermore, at a sub-lethal dose of 5ug/kg/hour the data suggests some level of recovery in WBC count with electrical stimulation. Redacted

Phase II: Evaluating splenic nerve stimulation in the porcine sepsis model

- *Surgical feasibility was confirmed but lead complications restricted the sample size and confirmation of efficacy.*

Instead of delivering the stimulation after the LPS administration as was done in Phase I. In phase II, electrical stimulation was initiated at the same time LPS administration was initiated. After 2 hours, the LPS was turned off and electrical stimulation continued for another two hours (except where noted). Table 1 outlines the various procedures, animal ID for further reference, and the outcome. Efficacy was noted in two of the procedures (9/9/03 &

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only those procedures that were similar in methods and parameters were selected for further analysis in the graphs below.

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Table 1. List of procedures.

Procedure Date	**Animal ID**	**Experimental Group**	**Procedure Notes Of Significance**
Redacted	82	**Pilot**	Establish model (50ug/ml/hr LPS)-See appendix B
	200	**Pilot**	Establish model (25ug/ml/hr LPS)
3/25/03	196	**Pilot**	Establish model (5ug/ml/hr LPS)
6/11/2003	334	**LPS alone**	See appendix C
6/27/2003	321	**LPS alone**	
7/3/2003	402	**LPS alone**	Drug infusion system malfunctioned. Actual LPS dose = 6.1 ml versus 6.85 ml.
7/11/2003	409	**LPS alone + cut splenic nerve** (neg control for stim)	Chased with dobutamine
7/29/2003	490	**Stimulation alone, No LPS.** (neg control for LPS)	Altered stim parameter sets: 90usec or 450usec, 1V or 10V, 10 hz or 100hz. for 10 minutes, changed every hour. Increased WBC count over baseline.
9/9/2003	548	**LPS + Stimulation** (450 usec,10mAmps,10hz)	Rescued LPS induced WBC count and prevented cytokine upregulation.
	561	**LPS + Stimulation** (450 usec, 10mAmps, 10hz)	
	616	LPS + Stimulation (450 usec, 10mAmps, 10hz)	
	712	LPS + Stimulation (450 usec, 10mAmps, 10hz)	
	834	**LPS + Stimulation** (450 usec, 10mAmps, 10hz)	Redacted
Redacted	875	**LPS + Stimulation** 450usec, 120 hz	
	877	**LPS + Stimulation** with various parameters.	
	827	**LPS + Stimulation** Altered stim from 25-50 mAmps. Used paddle electrode.	
	954	**Prophylactic stimulation** (90 min), LPS + Stimulation.	

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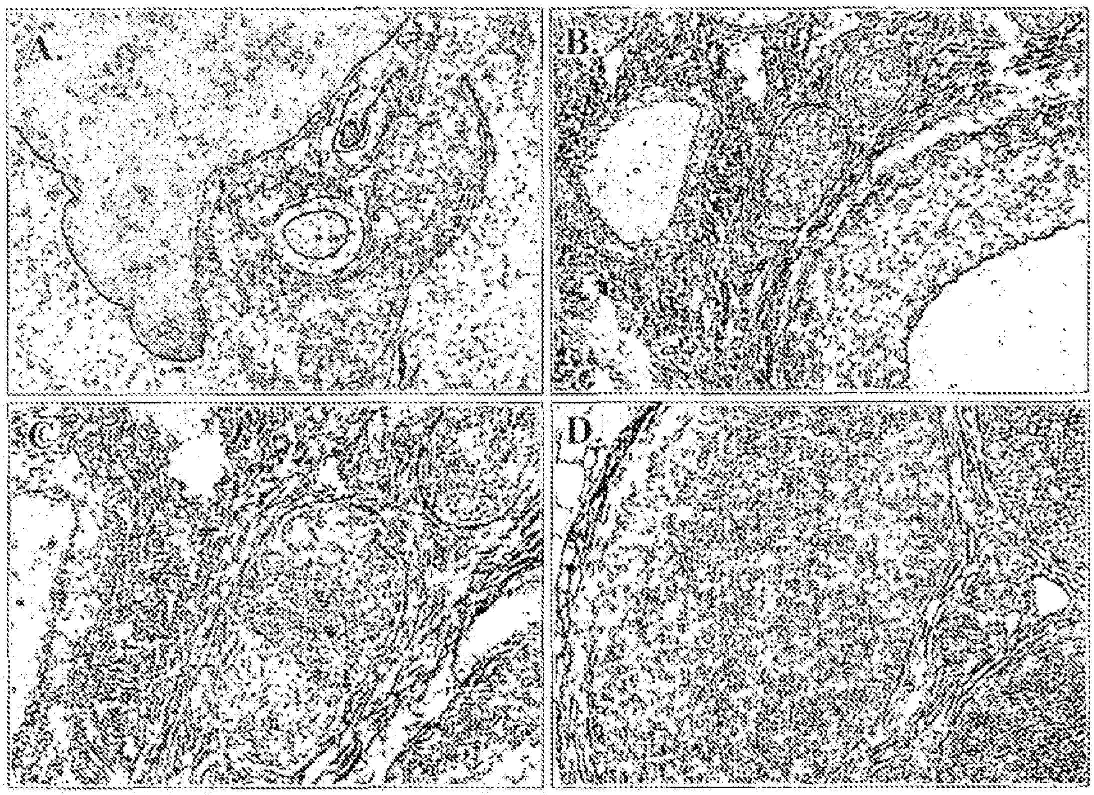
- *Pathology notes of interest.*

Tissue samples taken proximal and distal to the electrode included profiles of nerve, artery and vein and were generally within normal limits. In some procedures, the tissue beneath the electrode was within normal limits while in other procedures mild pathology was evident. Some of the changes noted multifocally within nerves include nuclear pkynosis, occasional nuclear debris, vacuolation, and occasional presence of neutrophils. Of these, vacuolation was the most frequently observed evidence of pathology (Fig. 6). See achieved pathology reports

Figure 6. Histopathology. Sections of nerve taken underneath the electrode following LPS infusion and 6 hours of stimulation. A-C) Presence of neutrophils and mild vacuolation in the nerve tissue. D) Animal #548 exhibiting dark, pyknotic nuclei.



- *There are no negative effects of SNS on cardiac physiology.*

The results of Phase I with respect to cardiac physiology were confirmed in Phase II in that SNS produces no adverse effects on cardiac physiology. Broader ranges of parameters were tested in Phase II and there were no observable negative effects on arterial pressure or cardiac output. The mean arterial blood pressure (MABP) is expected to drop as a result of the systemic immune response due to LPS adminisiration. Therefore, the sepsis model is further validated since this effect was observed.

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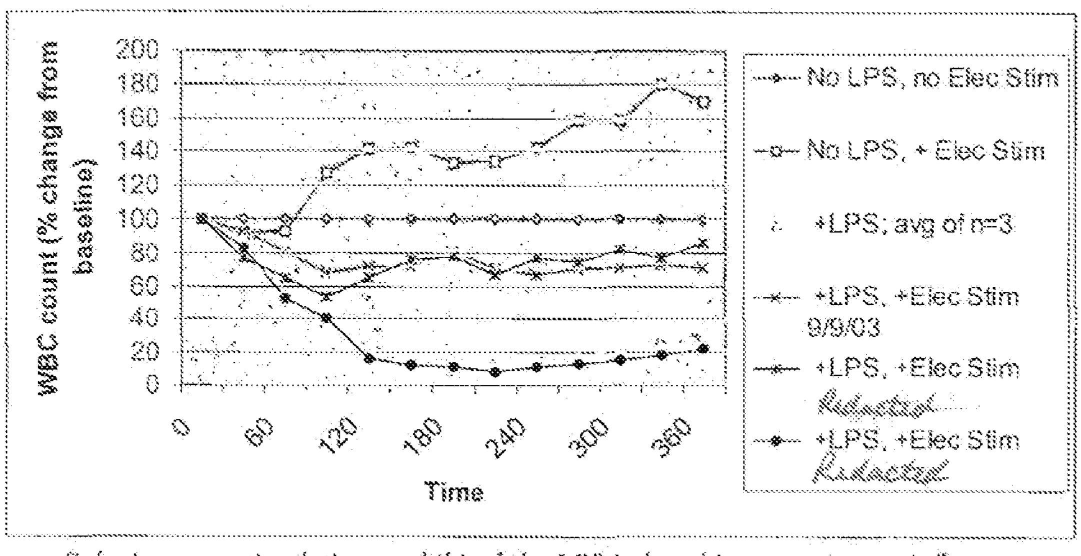
• *Blood chemistry (white blood cell count and lactate levels) confirms sepsis model.*
The lactate levels increased following initiation of LPS administration. The range varied per procedure but generally went from ~2.1-5.3 mmol/L. Additionally, the white blood cell count decreased following LPS administration when electrical stimulation is not applied. Increasing lactate levels and decreasing white blood cells are both indicators of an early sepsis response.

• *Splenic nerve stimulation maintained the white blood cell count (n=2) suggesting efficacy.*
In the presence of systemic LPS, the white blood cell count drops significantly. In two procedures electrical stimulation of the splenic nerve rescued the WBC count and maintained it at about 75% of baseline (Fig. 7). However, in other procedures this same effect was not observed. The slight drop in WBC count in the two efficacious procedures confirms that LPS was indeed delivered, since the initial counts drop below baseline. The WBC count served as an early indicator of success/efficacy. If the WBC count continued to show a decline at 180 minutes, the stimulation parameters were often altered to attempt some moderate effect. Interestingly, electrical stimulation alone results in an enhanced WBC count.

Figure 7. White blood cell (WBC) count.



• *Splenic nerve stimulation prohibited the LPS induced increase in pro-inflammatory cytokines (n=2) suggesting efficacy.*
It is widely understood that in the presence of systemic LPS, pro-inflammatory cytokines (TNFα, IL-1 and IL-6) increase.

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In the animals receiving LPS alone or those where the SNS was ineffective, the cytokine levels are significantly enhanced compared to those that either did not receive LPS or those where the SNS was

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effective (Fig. 8). An increase in these cytokines in the 'LPS alone' controls is further confirmation of a valid sepsis model and also serves as Redacted significant endpoint measurement. The baseline levels of TNFα, IL-1 and IL-6 in the pig are nearly equivalent to those observed in humans. No IL-1 or IL-6 is observed without the LPS challenge here and in other sepsis models.

The two procedures dated 9/9/03 and Redacted demonstrate that the addition of SNS can ameliorate all three LPS induced pro-inflammatory cytokine levels (Fig. 8 A-C). The results of these two procedures suggest potential for therapeutic efficacy since cytokine levels are held at baseline.

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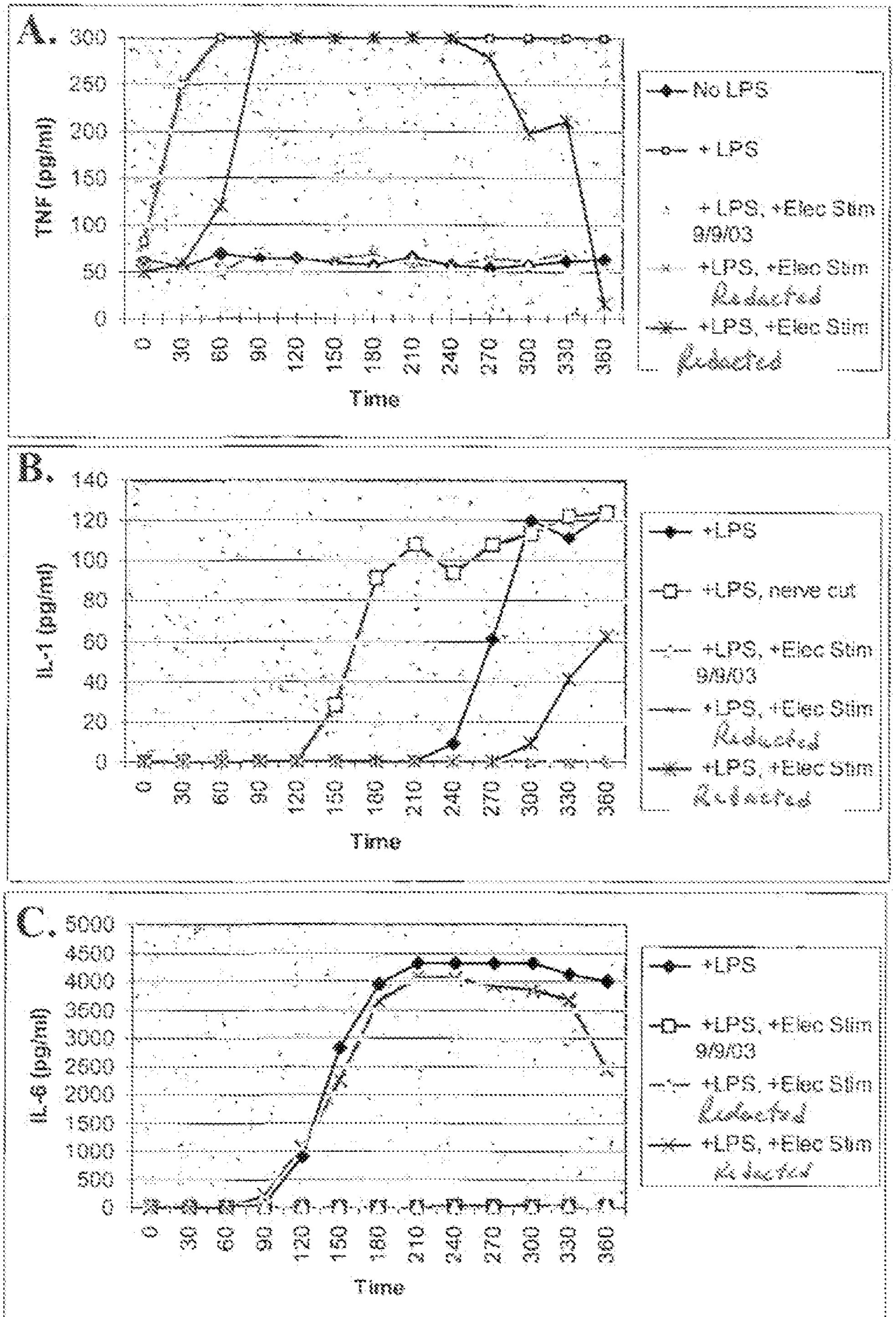
Figure 8. Cytokine response. A) Serum levels of Tumor Necrosis Factor, B) Serum levels of interleukin-1beta, C) Serum levels of interleukin-6.



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VI. DISCUSSION

Establishing a porcine sepsis model.

This study successfully established an acute large animal sepsis model that reproduces the experimental condition of the standard rodent sepsis models and allows for the testing of medical

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Safety of electrical stimulation to the splenic nerve.

The data demonstrate that electrical stimulation of the splenic nerve is safe in that there is no adverse effect on cardiac physiology.

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Therapeutic efficacy of electrical stimulation of the splenic nerve.

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the results suggest that electrical stimulation of the splenic nerve down-regulates systemic TNF production and the development of shock and inflammatory sequlea during lethal endotoxemia.

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Significance& rationale for this therapeutic approach.

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Future studies.

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VII. REFERENCES

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Final Report
Study #: 0001E0007

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APPENDIX A: Procedure #1 report

Splenic Nerve Stimulation
Report on pig #1
SN# 0001E007

Summary

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Purpose

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Method

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VIII. Results

A. Evaluate the feasibility of splenic nerve stimulation.

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B. Establish an effective dose of LPS to generate a septic swine model.

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C. Determine the effect of acute stimulation on the LPS induced inflammatory response, and blood chemistries.

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D. Evaluate any physiological/pathological changes resulting from the LPS or stimulation.

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V. Conclusions

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